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Solid State Fermentation Production of Cellulase from *Bacillus* sp.

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ABSTRACT:
Bacillus sp. was cultured in solid-state fermentation (SSF) of wheat straw to produce cellulase. The fermented biomass was harvested after 36 h of SSF at pH 8 and temperature 40^0C. It was filtered and centrifuged at 10,000 rpm at 4^0C and supernatant was collected as crude enzyme extract. Maximum activity of cellulase (3.775±0.13U/ml) was obtained after fermentation of wheat straw (10g) medium containing 0.2g soybean meal, 0.04g corn steep liquor (CSL), 80% moisture content (mineral salt medium, pH 8), 2-mL inoculum, and temperature 40^0C. SSF was found to be more productive than submerged fermentation (SmF) in terms of cellulase yields. The partial purification of cellulase was carried out through $(NH_4)_2SO_4$ precipitation. The partially purified enzyme produced under SSF had molecular weight of 35 and 45kDa. It was active in a broad pH (4-10) and temperature range (25-55^{0}C). The optimum, pH and temperature of *Bacillus* cellulase were pH 5 and 45^0C, respectively. At 50^0C and 60^0C, the half lives of the partially purified cellulase were 194 and 163 min, respectively. All the results indicated that the *Bacillus* sp. had a promising application of treatment of agro-wastes and cellulase from *Bacillus* sp. could be potentially used in biofuel industries.

Key Words: Cellulase; *Bacillus* sp.*;* Solid State Fermentation; Optimization; Partial purification

INTRODUCTION

Cellulases are among the industrially important hydrolytic enzymes and are of great significance in present day biotechnology. Cellulases are widely used in the food, feed, textile and pulp and paper industries [1].

In textile industry, these are used in biopolishing of cotton fabrics and to produce stone washed look of denim garments. Microbial conversion of cellulosic/ lignocellulosic biomass into useful products is a complex process involving combined action of three enzymes namely endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) [2]. Production of cellulases and their properties have been extensively studied during recent years [3].

The development of microbial strains, media composition and process control have all contributed to achievements of high levels of extra cellular accumulation of cellulases for subsequent applications in industrial processes [4].

Wheat straw abundantly available in wheat production fields and markets appears to be a favorable substrate as it is cheaply available in the tropical and subtropical countries and has a cellulose content of 35-40% [5]. SSF offers advantages over fermentation in liquid broth (submerged fermentation) like higher product yield, better product quality, cheaper product recovery and cheaper technology [6-7]. This paper reports the optimization of SSF process for cellulase production by *Bacillus* sp. grown on wheat straw and its characterizations.

MATERIALS AND METHODS

Substrate

What straw used as substrate for cellulase production was obtained from field, Nasik, India. Substrate was blend uniformly in blender to form powder.

Fermentation organism and inoculum

Pure culture of *Bacillus* sp. a soil isolate isolated from garden soil Nasik. It was maintained on Nutrient agar slants and glycerol stocks and preserved at 4 and -20^0C, respectively. The defined inoculum mineral salt medium contain (g/L); glucose 20, NaCl 5, KH_2PO_4 2.5, K_2HPO_4 3, $MgSO_4$ 0.2 (pH 8). Nitrogen sources soybean meal 0.1g and corn steep liquor 0.01g were supplemented separately in Wheat straw. An inoculum was prepared by inoculating Nutrient broth by loopful culture from agar slant.

Solid state fermentation and enzyme extraction

The fermentation flasks containing 10 g wheat straw moisturized with the mineral salts medium (exclude soybean meal and CSL were autoclaved and inoculated with 1 ml inoculum (10^8cells/ml). The fermentation flasks were incubated at 35^0C for 60h (unless otherwise indicated) without shaking for solid state fermentation.

Enzyme extraction

The enzyme was extracted by adding 100 ml of 100mM citrate buffer (pH 5) to the fermented medium and incubated for 60 min under shaking (150rpm) condition. After incubation, whole mixture was filtered through Whatman No. 1 filter paper. The filtrates were centrifuged at 5000 rpm to remove bacterial cells and

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spores and supernatant was used as crude enzyme extract.

Enzyme assay

The cellulase activity was determined by the assay method of Dutta *et al.*, [8]. The supernatant containing cellulase enzymes (0.5 ml) was incubated with 0.5 ml of CMC (1 %) in citrate buffer (50mM, pH 5) for 20 min at 40^0C. The reducing sugars released were measured with 3,5-dinitrosalicyclic acid reagent using glucose as a standard [9]. One unit (U) of cellulase activity was defined as the amount of enzyme releasing one µmole of glucose equivalent per minute under the assay conditions.

Production Optimization

Growth medium of wheat straw moistened with varying volumes of mineral salts medium (different pH) was fermented with *Bacillus* sp. for different fermentation periods with varying inoculum size. The effect of varying concentrations of soybean meal and CSL was also investigated. The level of a parameter optimized in an experiment was maintained in the subsequent studies.

Comparison of SSF and SmF

To compare SmF and SSF, the SmF medium was prepared in duplicate by adding 5 g of wheat straw in 100 ml optimum growth medium. After autoclaving and inoculation the SmF flasks were incubated on at 40^0C for 60h under continuous shaking (150 rpm). The SSF flasks containing optimum growth medium were processed as described earlier.

Partial purification and characterization of cellulase

The crude cellulase of *Bacillus* sp. was partially purified by ammonium sulfate precipitation (60% saturation) under cold conditions and dialyzed against 50mM citrate buffer (pH 5). The partially purified enzyme was subjected to denaturation (SDS-PAGE) on 10% (w/v) gels by the method of Holt and Hartman [10]. After electrophoresis, the gels were stained for protein with Comassie Brilliant Blue R250 [11]. Molecular weight standards purchased from Sigma (St Louis, MO, USA) of α-lactalbumin (14.2 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), albumin (66 kDa) and phosphorylase B (97 kDa) were used. Gel electrophoresis on 1% CMC (w/v) was run and analyzed by zymogram analysis [10]. Gels were stained for cellulase activity in a Congo Red solution (0.1%, w/v) at room temperature for 30 min. The activity band was observed as a clear colorless area, depleted of CMC, against a red background when destained in 1M NaCl solution.

The partially purified enzyme of *Bacillus* sp. was characterized with respect to its activity under different pH (4-10) and temperature (25-55^0C) conditions. The thermostability of the enzyme was determined at 40-70^0C for up to 180 min.

RESULTS AND DISCUSSION

Fermentation parameters were optimized for production of cellulase by *Bacillus* sp. in SSF medium of wheat straw and results have been discussed as under:

Fermentation period

SSF medium of wheat straw was inoculated and incubated at pH 7 and 35^0C and duplicate flasks were processed for different time periods.

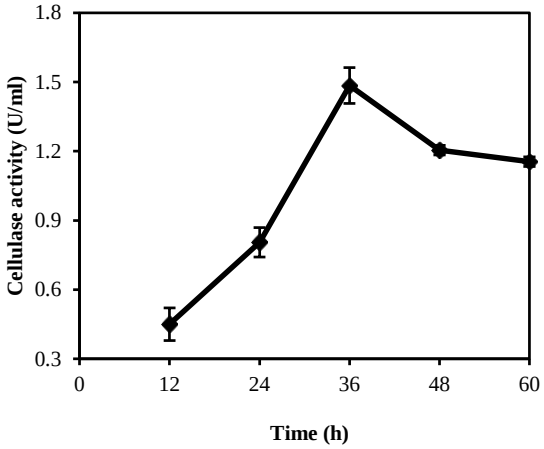

Fig. 1. Effect of fermentation time on cellulase production by *Bacillus* sp. in SSF of wheat straw, soybean meal 0.1g, corn steep liquor 0.01g, initial moisture 50% (v/w), pH 7, 35^0C, 1 ml inoculum. Error bar represents the standard deviation (±) mean of triplicate analysis.

The cellulase activity increased up to 36h (1.485±0.07U/ml) of incubation and decreased, thereafter (Fig. 1). Romero *et al*., found the maximum activity of cellulase at 36h but after that a steep decrease was observed by increasing the fermentation time [12].

Moisture level

The experiment was carried out to study the effect of varying moisture content. Maximum cellulase (1.82±0.09U/ml) production by *Bacillus* sp. was observed with 80% moisture (Fig.2). The results indicated that when moisture level increased beyond a certain limit the enzyme activity started decreasing. This decline may be attributed to poor aeration in SSF and partial adsorption of enzyme to the substrate. Xia *et al*., studied the cellulase production by solid state fermentation on lignocellulosic waste and reported that water content of solid substrate is one of the key factors in cellulase production experiments. SSF at a water content of 70% was found to be the most suitable for cellulase production [13].

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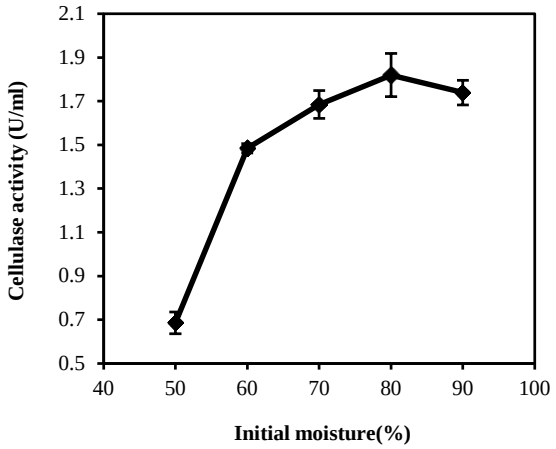

Fig. 2. Effect of initial moisture on cellulase production by *Bacillus* sp. in SSF of wheat straw, soybean meal 0.1g, corn steep liquor 0.01g, pH 7, 35^{0}C, 1 ml inoculum, 36h. Error bar represents the standard deviation (±) mean of triplicate analysis.

Inoculum size

There was a gradual increase in enzyme production by *Bacillus* sp. by increasing inoculum size and optimum cellulase (2.175±0.04U/ml) was recorded in the medium receiving 2 ml inoculum under pre-optimized conditions. A further increase of inoculum up to (3-5 ml) showed decreasing trend (Fig. 3). Maximum exoglucanase activity (3.52U/ml) was reported by 5% inoculum of *Bacillus subtilis* on SSF of banana stalk [14]. Results of our study are in line with those of Zhang *et al*., who investigated the effect of inoculum size on cellulase synthesis by *Trichoderma viride* and described that the impact of the amount of inoculant on cellulase production was small and 5% inoculum was the most suitable [15].

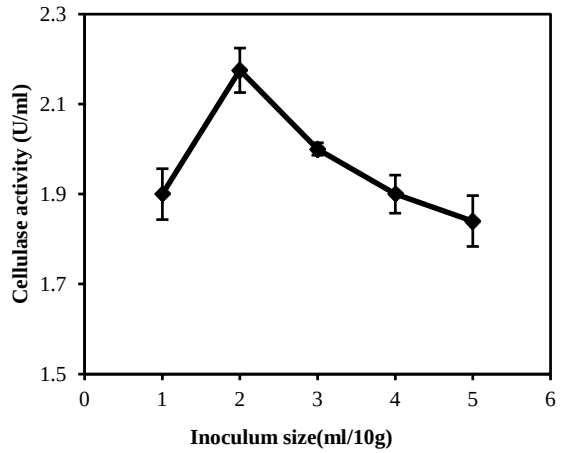

Fig. 3. Effect of inoculum size on cellulase production by *Bacillus* sp. in SSF of wheat straw, soybean meal 0.1g, corn steep liquor 0.01g, initial moisture 80% (v/w), pH 7, 35^{0}C, 1 ml inoculum, 36h. Error bar represents the standard deviation (±) mean of triplicate analysis.

Effect of pH on cellulase production

The SSF process is known to be influenced by the pH of environment. In confirmation, our results (Fig. 4) clearly indicate that the optimal production of cellulase by *Bacillus* sp. is pH-dependant. A maximum cellulase activity of 2.8±0.07U/ml was obtained at initial pH of 5.

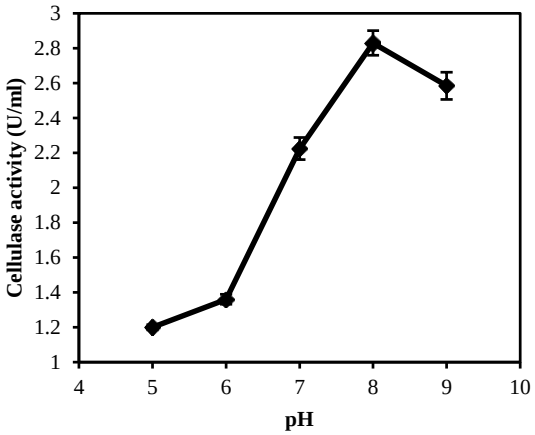

Fig. 4. Effect of initial pH on cellulase production by *Bacillus* sp. in SSF of wheat straw, soybean meal 0.1g, corn steep liquor 0.01g, initial moisture 80% (v/w), 35^{0}C, 2 ml inoculum, 36h. Error bar represents the standard deviation (±) mean of triplicate analysis.

The use of the pH control to provide suitable conditions for optimum enzyme production in SSF was widely reported in literature. Obtained data confirmed the findings reported by Ray *et al*. who mentioned that pH 7–7.5 more suitable for optimization of cellulase product ion by *B. subtilis* and *B. circulans* [16]. Furthermore, the cellulolytic enzyme, endogluconase obtained from *Cellulomonas*, *Bacillus*, and *Micrococcus* spp. hydrolyzed substrate in the pH range of 4.0 to 9.0, with maximum activity transpiring at pH 7 [17].

Effect of Temperature on cellulase production

The resultant temperature in a SSF system is determined by both the environment temperature and that generated from the metabolic activities of the growing microorganisms. In our work, the cellulase production by *Bacillus* sp. peaked (2.39±0.08 U/ml) at 45^{0}C (Fig. 5). Notably, the enzyme production declined at temperatures above and below 45^{0}C with only 3.155±0.03 U/ml, at 40^{0}C, and 1.03±0.07 U/ml, at 50^{0}C. These results are similar to those of Immanuel *et al*., found that the cellulase enzyme produced by *Cellulomonas*, *Bacillus* and *Micrococcus* spp. at 40°C [17]. Ray *et al*., also recorded maximum cellulase produced by *B. subtilis* and *B. circulans* at 45°C [16].

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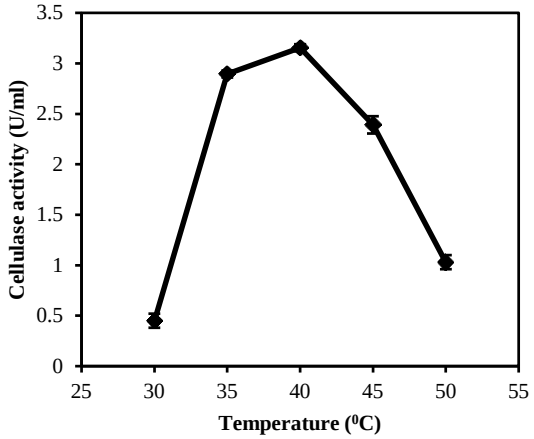

Fig. 5. Effect of incubation temperature on cellulase production by *Bacillus* sp. in SSF of wheat straw, soybean meal 0.1g, corn steep liquor 0.01g, initial moisture 80% (v/w), pH 8, 2 ml inoculum, 36h. Error bar represents the standard deviation (±) mean of triplicate analysis.

Soybean meal

Effect of various concentrations of soybean meal was checked on cellulase production by *Bacillus* sp. and maximum cellulase 3.435±0.06U/ml was produced at 0.2g soybean meal (Fig. 6). Our results are in contrast with the work of Enari *et al*., who reported that good cellulase production can be obtained with the organic nitrogen sources such as yeast extract and peptone [18]. There were no reports on cellulase production with soybean meal but Heck *et al.*, reported cellulase and xylanases production from Amazon *Bacillus* strains using soybean industrial residue based solid-state cultivation [19].

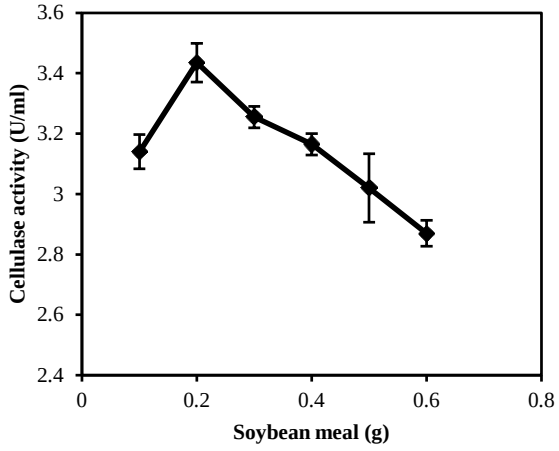

Fig. 6. Effect of soybean meal concentration on cellulase production by *Bacillus* sp. in SSF of wheat straw, corn steep liquor 0.01g, initial moisture 80% (v/w), pH 8, 40^{0}C, 2 ml inoculum, 36h. Error bar represents the standard deviation (±) mean of triplicate analysis.

Corn steep liquor (CSL)

Cellulase production by *Bacillus* sp. was studied with varying concentrations of CSL in the preoptimized wheat straw medium.

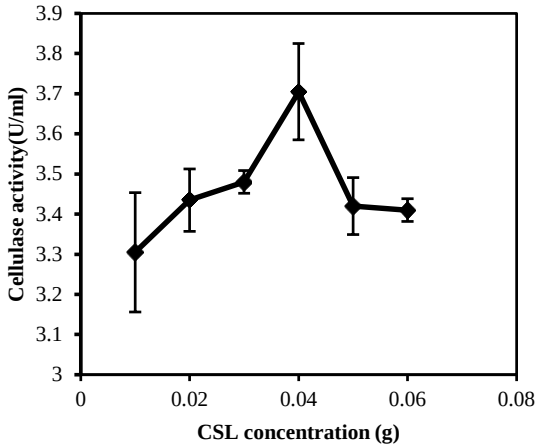

Fig. 7. Effect of CSL concentration on cellulase production by *Bacillus* sp. in SSF of wheat straw, soybean meal 0.2g, initial moisture 80% (v/w), pH 8, 40^{0}C, 2 ml inoculum, 36h. Error bar represents the standard deviation (±) mean of triplicate analysis.

An enhanced enzyme production by the bacteria was observed with the addition of CSL. Maximum activity of cellulase (3.705±0.12U/ml) was observed at 0.04% concentration as shown in Fig. 7 and with further increase of CSL, the yield of enzyme was decreased. When CSL was added (3%) to wheat bran medium for isolate 2, the CMCase production (2.26U/ml) was observed at pH 7 [20].

Comparison of SSF with SmF

Duplicate flasks containing the optimum growth medium for cellulase production were subjected to SSF and SmF for 60h. Results given in Table 1 indicated that SSF showed more production of cellulase (3.775±0.13U/ml) as compared to SmF (0.72±0.08U/ml). Murthy *et al*. described that SSF involves the growth of microorganisms on solid substrate and through SSF process cellulase can be produced on a large scale with high productivity and uniform quality [21]. Exoglucanase production was also observed by *Bacillus subtilis* 2.6 and 0.85U/ml by SSF and liquid state fermentation (LSF), respectively [14].

Table 1. Comparative cellulase production

Fermentation Type[a]	Cellulase activity (U/ml)
SSF[b]	3.775 ±0.13
SmF[c]	0.72±0.08

[a]Optimum condition, soybean meal 0.2g, corn steep liquor 0.02g, pH 8, 40^{0}C, 2ml inoculum, 36h,
[b]SSF, solid state fermentation with 10g wheat straw, initial moisture 80% v/w)
[c]SmF, submerged fermentation with 5 g wheat straw

Partial Purification and characterization

The partially purified enzyme of *Bacillus* sp. migrated on SDS-PAGE as several bands with different molecular

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weights (Fig. 8). Zymogram analysis of the crude enzyme revealed two bands staining for cellulase activity where a clear hydrolytic activity zone was formed against a dark background. Both bands were migrated with molecular masses of 45 kDa and 35 kDa, respectively. Mawadza *et al.*, reported a molecular weight of 40 kDa for cellulase from *Bacillus sp.* CH43 and HR68 [22]. Molecular weight (33kDa) of cellulase as monomeric nature from *B. brevis* vs-1 was reported by Singh and Kumar [23]. Here, we report on isozymes of 35 and 45 kDa cellulase from *Bacillus* sp.

The partially purified cellulase was active in the pH range 4-10 (optimum pH 5) and temperature range 25-55ºC with a temperature optimum of 45^{0}C (Fig. 9). Robson and Chambliss, published a report on a cellulase from *Bacillus* sp. which had an optimum pH and temperature of 4.8 and 58^{0}C, respectively [24].

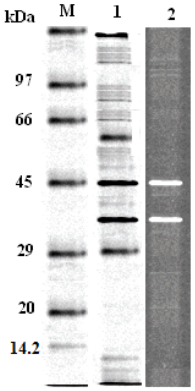

Fig. 8. SDS-PAGE of crude cellulase from *Bacillus* sp. (M, molecular weight markers; 1, crude cellulase; 2, zymogram of cellulase with CMC; molecular weight proteins: α-lactalbumin, 14.2 kDa; trypsin inhibitor, 20 kDa; carbonic anhydrase, 29 kDa; ovalbumin, 45 kDa; albumin, 66 kDa; phosphorylase B, 97kDa)

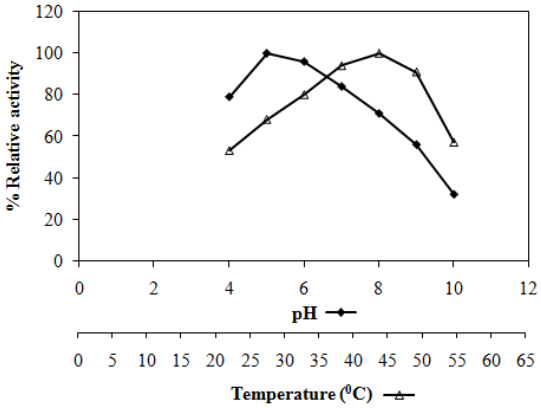

Fig. 9. Effect of pH (at 40^{0}C) and temperature (at pH 5) on activity of partially purified cellulase by *Bacillus* sp. with CMC as substrate (relative activity expressed as percentage of maximum activity)

The *Bacillus* sp. cellulase retained 60% of cellulase activity after incubation at 50ºC for 180 min (Fig. 10). The residual activities of *Bacillus* sp. cellulase after 60 and 180 min at 60^{0}C (96 and 40%) and 70^{0}C (90 and 0%) were reported respectively. At 50^o and 60^{o}C, the half-life of the enzyme was 194 min and 163min, respectively. Cellulase of *Bacillus* sp. VG1 showed half life of 12 min at 100^{0}C [25]. The thermostability of cellulase by *A. niger* Z10 was carried out in temperature range of 40-90^{0}C and retained 41.2% of its original activity at 90^{0}C for 15 min [26].

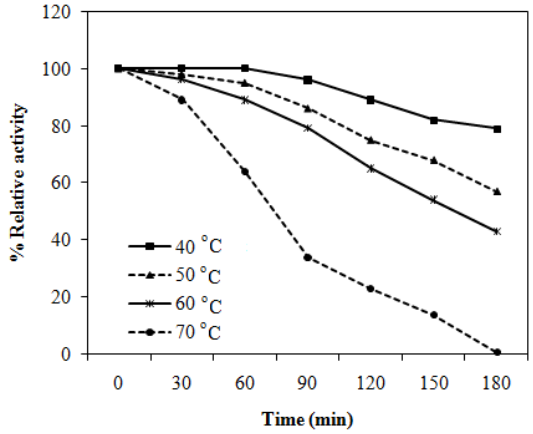

Fig. 10. Thermostability of partially purified cellulase by *Bacillus* sp. (relative activity expressed as percentage of maximum activity)

CONCLUSIONS

The present study clearly indicates that *Bacillus* sp. has potential of cellulase and was improved (2.54-fold) by traditional optimization method on inexpensive and easily available afro substrates. Soybean and CSL were responsible for induced cellulase production. SSF showed 5.24-fold higher cellulase production compared to SmF. Thus, it has potential application of biofuel and textile industries.

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